(–)-Gossypol Reduces Invasiveness in Metastatic Prostate Cancer Cells

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Abstract. *Background: Acquisition of metastatic ability by prostatic cancer cells is the most lethal aspect of prostatic cancer progression. (–)-Gossypol, a polyphenolic compound present in cottonseeds, possesses anti-proliferative and pro-apoptotic effects in various cancer cells. Materials and Methods: In this study, the differences between MAT-LyLu, rat prostate cancer cells, with a novel isolated subline from metastasized tumors in the lungs of MAT-LyLu-bearing Copenhagen rats (MLL cells) were compared with respect to cell growth and invasion. The effects of (–)-gossypol on cell viability, colony formation, invasive ability and cell migration in MAT-LyLu and MLL cells were also evaluated. Results: Results showed that MLL cells displayed higher growth ability, colony formation and aggressive penetration than those of MAT-LyLu cells. MLL cells possess lower protein expression of Bcl-xL and nm23-H1 than those of MAT-LyLu cells, implying differences in invasive ability. Moreover, (–)-gossypol treatment induced a dose-dependent inhibition of invasive activity and cell viability and reduced Bcl-2 and Bcl-xL proteins but induced nm23-H1 protein in both cell lines. Conclusion: These findings illustrated that (–)-gossypol reduced in vitro invasion of both the parental MAT-LyLu cells and the isolated MLL cells, suggesting that (–)-gossypol might serve as a chemotherapeutic and/or chemopreventive agent.*

Cancer is a major public health problem in the United States and other developed countries. Currently, one in four deaths is due to cancer. In men, prostate cancer is the most commonly diagnosed cancer after skin cancer in the United States (1). Prostate cancer can be eliminated by surgery or radiation if diagnosed at an early stage, but the cancer procurement of metastatic ability leads to clinically incurable disease. Although new therapeutic treatments could improve patient survival, cancer-related morbidity and mortality still occur.

Metastasis is the spread of cancer from the primary site of the body to another organ. The metastatic tumor contains cells that are like those in the original (primary) tumor (2). Prostate cancer originates as a localized lesion. It subsequently progresses to acquire increased invasive, migratory and metastatic potentials. Eventually, androgen independence develops and the tumor becomes refractory to conventional interventional therapies. The progression of clinical prostate disease from a localized tumor to a widely disseminated disease state probably occurs through alterations in proliferation, cell-cell adhesion, invasion potential and critical interactions between prostate cancer cells and surrounding host cells at both primary and metastatic tumor sites (2).

Metastasis-suppressor genes are identified by comparing the highly metastatic with poorly or non-metastatic tumor cells. Following re-expression of a metastasis-suppressor gene in a tumor cell, metastasis is inhibited but tumorigenesis is not significantly reduced (3). Currently, there are eight metastasis-suppressor genes named and among them, *nm23* is the first metastasis suppressor gene known. Nm23 has kinase activity and is recognized as a nucleoside diphosphate (NDP) kinase (3). Eight human *nm23* genes have been characterized so far and this gene possesses metastasis suppressive activity in a variety of tumor cell types. *In vitro* correlates of suppression include reduced invasion, motility and soft agar colonization and induction of differentiation (3). The first identified nm23 gene is nm23-H1. Clinical studies of nm23-H1 are abundant and commonly show down-regulation during the progression to metastasis for various types of cancer, including breast, melanoma, ovarian, hepatocellular, esophageal, oral squamous and laryngeal cancer (2).

In prostate cancer, *nm23-H1* may function as a metastasis suppressor gene because expression is lost in more than 80% of metastases, although it is present in 100% of primary

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lesions (4). It has also been found that reduced nm23-H1 expression is a predictor of prostate tumor stage and grade (4, 5), although this finding has been challenged in other reports (6, 7). There is some evidence that nm23-H1 can suppress metastatic colonization of DU-145 prostate cancer cells *in vitro* (8).

Gossypol is a polyphenolic pigment present in cottonseeds and in cotton plant by-products such as cottonseed oil and cottonseed meal flour. Results have showed that (–)-gossypol is a more potent inhibitor than (+)-gossypol and (±)-racemic gossypol (9, 10). A previous study (11) indicated (±)-gossypol reduced tumor mass and weight in (±)-gossypol-treated Copenhagen rats which was derived originally from the Dunning R3327H prostate cancer cell line and is one of the few models to study the metastatic process of prostate cancer. It was also found that (±)-gossypol inhibited metastasis into lymph nodes and lung in gossypol-treated rats which had higher gossypol concentration in serum (11). Our recent work isolated a subline from the metastasized lungs of MAT-LyLu-bearing Copenhagen rats, which was MLL cell line (12). MLL cells display a higher penetration percentage than MAT-LyLu cells in *in vitro* invasion assay and possess lower steady state levels of nm23 mRNA than MAT-LyLu cells (12). Both MAT-LyLu and MLL cells were susceptible to (±)-gossypol, which induced dose-dependent inhibition of invasive activity (12).

In this study, the differences between MAT-LyLu and MLL cells and the effects of (–)-gossypol treatment on cell viability, invasion and cell migration were further compared. Also, the regulation of anti-apoptotic Bcl-2 and Bcl-xL and anti-metastatic *nm23-H1* gene by (–)-gossypol was investigated in order to understand the roles of (–)-gossypol in tumor metastasis. The study focuses on (–)-gossypol-mediated growth and tumor inhibition by using two cell lines that are highly metastatic and these findings might suggest that (–)-gossypol may have cancer-chemopreventive as well as cancer-chemotherapeutic effects against prostate tumor metastasis.

Materials and Methods

Cell culture and reagents. MAT-LyLu cells were originally obtained through Johns Hopkins University and described in a previous article (12). MLL cells were an isolated subline of the MAT-LyLu cell line from the metastasized lungs of MAT-LyLu-bearing Copenhagen rats and isolated through procedures as previously described (12). Both cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing an antibiotic-antimycotic mixture (100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin) (Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS; Invitrogen) in a humidified incubator (37˚C, 5% CO_2 and 95% air).

Antibodies against Bad, Bak, Bax, Bcl-2, Bcl-xL, cyclin-D1, cyclin-D3, Cdk4, Cdk6, Phospho-Cdc2 and p27 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies against nm23-H1 and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidase (HRP) conjugates were from GE Healthcare (Piscataway, NJ, USA). (–)-Gossypol was provided by Dr. Michael Dowd from the USDA, Southern Regional Research Center (New Orleans, LA) and (–)-gossypol preparation was described in detail previously (13).

Doubling time assay. MAT-LyLu and MLL cells were plated separately at a density of 5,000 in 24-well plates in a volume of 1 mL/well. At the same time, a group of cells were counted as time 0 hour. Cells were grown for 3 days and counted every 12 hours. Adherent cells were detached by rapid trypsinization. An adequate volume of medium containing 50 % trypan blue was added and then cells were counted using a hemacytometer. Experiments were performed in 4 replicate culture wells for each group and each experiment was repeated twice. Based on the counted cell numbers at different time points a cell proliferation curve was generated. Cell doubling (CD) was calculated using the formula ln (Nj-Ni)/ln 2 where Nj or Ni are the cell numbers at different time points Tj or Ti (Tj>Ti) in the growth log phase of the cells. Doubling time (DT) was consequently obtained by dividing the time interval (Tj>Ti) by CD (14).

Cell viability assay. The effect of (–)-gossypol on cell viability was assessed by CellTiter 96® Aqueous solution (Promega, Madison, WI, USA) assay in 96-well plates according to the manufacturer's instructions. A total of 4,000 cells/well were seeded and cultured in RPMI supplemented with 5% FBS overnight. Cells were treated with (–)-gossypol or 0.1% ethanol (vehicle control) in fresh medium with FBS for 24 hours. Briefly, at the end of treatment, cell viability was measured by adding a mix of 20 μL freshly combined MTS and PMS (the ratio at 20:1) solution to each well. The plates were incubated for 30 min and the color density was measured as the optical density at 490 nm (OD 490 nm) using an ELISA plate reader. Results were expressed as the ratio of viable treated cells to vehicle treated cells in a multiwell plate format.

Soft agar analysis for colony formation. The effect of (–)-gossypol on the colony formation of MAT-LyLu and MLL cells was examined by soft agar assay. MAT-LyLu and MLL cells were cultured in 6-well plates first covered with an agar layer (RPMI-1640 containing 0.5% agar and 5% FBS). The middle layer contained 1.5×10^4 cells/well in presence or absence of gossypol of RPMI-1640 medium containing 0.35% agar and 5% FBS. The top layer, consisting of 1 mL of medium, was added to prevent drying of the agar in the plates. The plates were incubated for 7 days. After incubation, the plates were stained in 0.5 mL of 0.005% crystal violet for >1 hour and the cultures were inspected and photographed using a microscope. Colony number was calculated as the average of colonies counted at ×40 magnification in five individual fields.

Cell migration assay. Tumor cell migration was assayed in transwell chambers (Costar®, polycarbonate membrane; Corning, Corning, NY, USA) according to the methods reported by Repesh (15) with some modifications. Briefly, transwell chambers with 6.5-mm polycarbonate filters of 8 μm pore size were used. After pre-incubation with (–)-gossypol for up to 24 hours, cells (2.5×10^5/mL) were finally suspended in RPMI-1640 medium (200 μL, serum free), placed in the upper transwell chamber. Sequentially, these chambers were incubated for 5 hours at 37˚C and then the cells on

the upper surface of the filter were completely wiped away with a cotton swab. The cells on the lower surface of the filter were counted under a microscope at ×200 magnification. For each replicate, the tumor cells in 3 randomly selected fields were determined, and the counts were averaged.

In vitro assay of invasive activity. The effect of (–)-gossypol on the invasive activity of MAT-LyLu and MLL cells was examined with a rapid *in vitro* assay as described (15) in the 24-well Transwell® method. Cell culture inserts (Costar) were converted into invasion chambers by applying a layer of reconstituted basement membrane coated with 100 μL RPMI containing Matrigel (BD Biosciences, San Jose, CA, USA) (at 20/1). Cell treatment and preparation were identified as cell migration assay. After incubation for 24 hours at 37˚C, the number of cells that had traversed the filter to the lower chamber was counted under a microscope at x200 magnification. For each replicate, the tumor cells in 3 randomly selected fields were determined, and the counts were averaged.

Cell movement. MAT-LyLu or MLL cells were plated at 5×10^5 cells per well in a 6-well plate and the higher density of cells allowing easier quantification of migration. After a 48-hour attachment period, the cells were wounded. The wound was scraped by using a sterile 200 μL micropipette tip into the confluent monolayer. The cultures were washed twice and then changed to new medium containing the appropriate concentration of (–)-gossypol. Cells were photographed per well along the wound at 24 hours later using an inverted phase contrast microscope at ×100 magnification.

Western blot analysis. Western blot analysis was carried out as described previouly (16). Lysates of MAT-LyLu and MLL cells were resolved on a 10% SDS-PAGE and then were transferred to a polyvinylidene difluoride (PVDF) membrane. The transblotted membrane was blocked with 10% nonfat milk for 1 hour and then incubated with the appropriate primary antibody at 4˚C overnight. All primary antibodies were diluted in 5% nonfat milk-containing PBS with 0.1% Tween-20 (PBST). Membranes were rinsed and washed briefly, followed by goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates for 1 hour at room temperature. The immunoblots were enhanced by chemiluminescence with an ECL Plus reagent (GE Healthcare, Piscataway, NJ, USA) and visualized by Fujifilm image system(FujiFilm, Medical Systems U.S.A., Stamford, CT, USA).

Statistical analysis. The results of cell viability assay and cell number were presented as the mean±standard deviation (s.d.) for three or six replicate culture wells as one group. Analysis was performed using StatView® ANOVA unpaired *t*-test. A probability (*P*) of less than 0.05 was considered to be statistically significant.

Results

Comparison of the growth rates and protein expressional characteristics in MAT-LyLu and MLL cells. To investigate the two cell lines, *in vitro* growth patterns, cell proliferation rates were measured over a 72-hour culture period. The trypan blue dye-exclusion method was used to evaluate the cell proliferation by doubling time. Results revealed that MLL cells had a greater growth rate than that of the parental MAT-LyLu cells. Doubling time of MAT-LyLu cells was 10.4 hours and that of MLL cells was 8.7 hours (Figure 1A).

The related proteins in cell cycle machinery and the Bcl-2 family were also investigated. Results showed that MLL cells had higher expression of cyclin-D1, cyclin-D3, CDK4 and CDK6 but lower expression of p27 compared with those of MAT-LyLu cells (Figure 1B). Moreover, MLL cells had higher expression of Bcl-2 protein, but lower expression of Bcl-xL and Bad protein than those of parental MAT-LyLu cells. There is a similar Bax expression between the two cell lines. Examination of the metastatic suppressor gene, *nm23-H1* was also performed. Data indicated that MLL cells had lower expression of nm23-H1 than that of MAT-LyLu cells (Figure 1B). Taking these findings together, MLL cells proliferated more rapidly than MAT-LyLu cells, which might be correlated with the higher expression of cell cycle machinery proteins, such as cyclin-D1 and cyclin-D3.

(–)-Gossypol reduced cell viability and colony formation in MAT-LyLu and MLL cells. (–)-Gossypol has been shown to induce a dose-dependent inhibition of cell proliferation in various cells (9-12, 16-17). To confirm anti-viability of (–)-gossypol in MAT-LyLu and MLL cells, both cell lines were subjected to (–)-gossypol in concentrations from 1 to 100 μM or 0.1% ethanol (vehicle control) for 24 hours. Findings showed that cell viability was significantly reduced by treatment of increasing (–)-gossypol concentrations in both cell lines. At 20 μM treatment, (–)-gossypol reduced cell viability by more than half compared with vehicle only. At 50 and 100 μM doses of (–)-gossypol, cell viability was almost reduced completely. Moreover, IC_{50} of both cell lines were similar at 14.5 μM. These results indicated that metastatic lung cells were sensitive to (–)-gossypol as well as their parental MAT-LyLu cells (Figure 2A).

In vitro colony formation was also used to evaluate the long-term cell viability and growth. MAT-LyLu and MLL cells were treated with 5 or 10 μM (–)-gossypol or 0.1% ethanol as vehicle control in soft agar. After 7 days incubation of vehicle-treated groups, MLL cells showed significantly more colony numbers and larger colonies than those of the parental MAT-LyLu cells. This result was consistent with cell growth rate which showed MLL cells had shorter doubling time than MAT-LyLu cells. The colony formation of MLL cells also confirmed that these isolated cells were a cancerous lung cell line. In (–)-gossypol treated groups, it was found that (–)-gossypol significantly inhibited colony number and size in both MAT-LyLu and MLL cells. It is worth noting that (–)-gossypol at 10 μM almost inhibited colony formation in both cell lines (Figures 2B, C).

(–)-Gossypol inhibited in vitro cell invasion in MAT-LyLu and MLL cells. Metastasis is the major step causing pain and mortality in cancer patients. To elucidate anti-

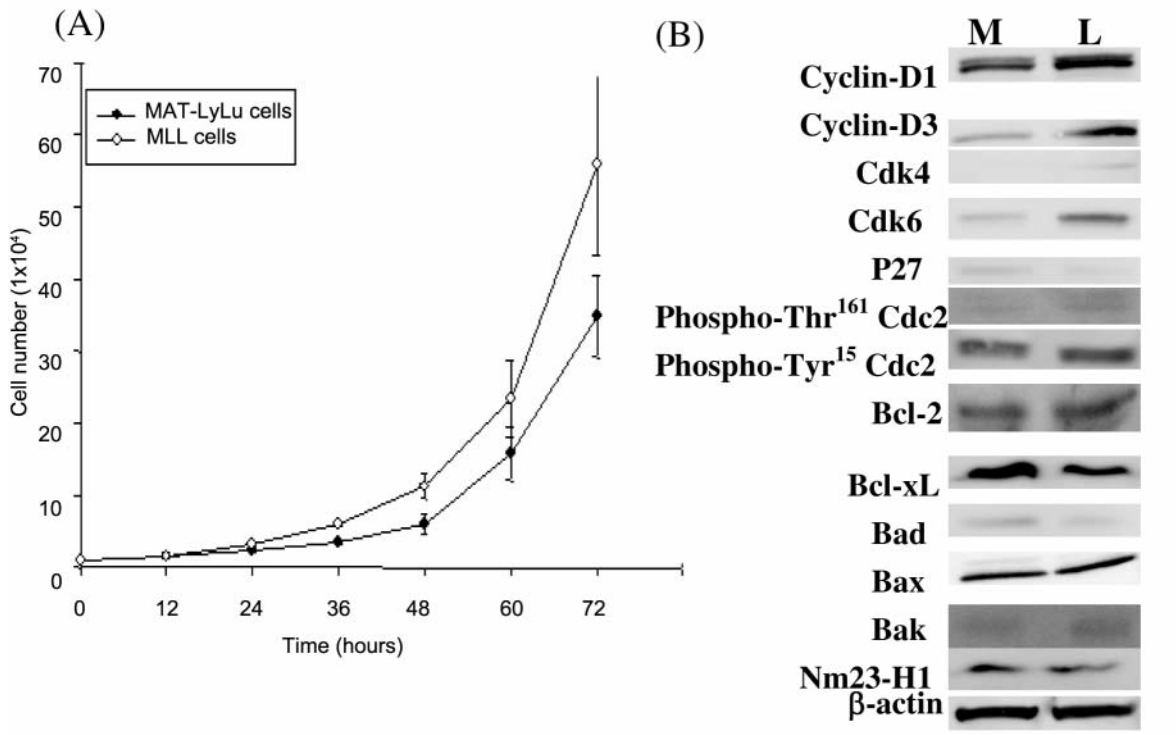

Figure 1. *Comparison of cell growth and protein characteristics of MAT-LyLu and MLL cells. (A) The growth of MAT-LyLu and MLL cells assessed by cell number calculation. Each bar represents the mean±SD of 4 wells. (B) Characterizations of protein expression between MAT-LyLu (M) and MLL (L).*

metastatic ability of (–)-gossypol in an *in vitro* invasion assay, cells were pre-treated with (–)-gossypol or vehicle for 12 or 24 hours. At the end of treatment, equal numbers of cells were put onto inserts coated with Matrigel. Following 24 hours, invaded cells were stained and counted. Data indicated that (–)-gossypol pre-treatment significantly reduced cell invasive ability in MAT-LyLu and MLL cells, and this inhibitory effect was in a dose-dependent manner. In vehicle-treated groups, MLL cells were more invasive than MAT-LyLu cells (Figures 3A, B). These data suggested that MLL cells were more aggressive than MAT-LyLu cells when grown on Matrigel and that (–)-gossypol was a potent metastatic inhibitor in prostate cancer cells.

(–)-Gossypol inhibited MAT-LyLu and MLL cell migration and movement. Cell movement is a critical point in cancerous metastasis. Cancer cells (MAT-LyLu and MLL) were pre-treated with or without (–)-gossypol for 12 or 24 hours. Equal number cells were seeded onto inserts and then allowed cell migration for 5 hours. At the end of each time point, cells on the top of membranes were removed by a cotton swab. Cells on the bottom of membranes were stained and counted under a microscope. It was found that (–)-gossypol pre-treatment significantly reduced cell migration in MAT-LyLu and MLL cells, and this inhibitory effect occured in a dose-dependent manner (Figure 4A). It is interesting to note that MAT-LyLu cells showed a higher migration rate than those of MLL cells, suggesting that Matrigel affected cancer cell migration.

To further identify anti-migration effect of (–)-gossypol, *in vitro* cell movement was employed using a cell wounding model. Briefly, after cancer cells grew to confluence, a scraped monolayer was created by using a micropipette tip. For the vehicle-treated groups, MAT-LyLu and MLL cells moved and recovered the free space. At 5 μM (–)-gossypol treatment, both MAT-LyLu and MLL cells showed cell movement inhibition (Figure 4B). These results indicated that (–)-gossypol could reduce cancer cell migration and movement.

(–)-Gossypol down-regulated Bcl-2 and Bcl-xL but up-regulated nm23-H1 in MAT-LyLu and MLL cells. To identify the molecular basis of (–)-gossypol-reduced cell growth and invasion in MAT-LyLu and MLL cells, a Western blot analysis was performed. Gossypol was reported to bind with Bcl-2 and Bcl-xL, which are related to anti-apoptosis. Previous reports (16, 17) found that (–)-gossypol treatment inhibited Bcl-2 and Bcl-xL protein expression in cancer cells. Therefore, examination of (–)-gossypol alteration of Bcl-2 and Bcl-xL expression in MAT-LyLu and MLL cells

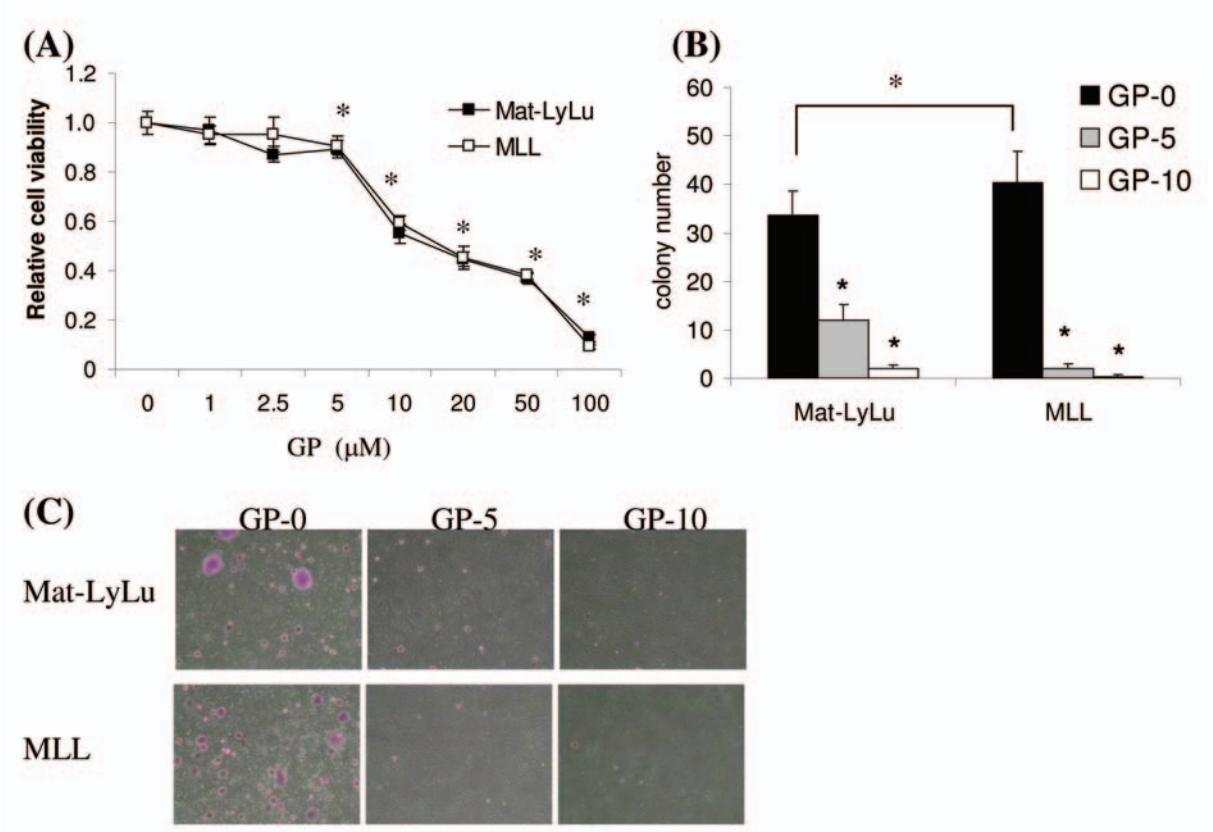

Figure 2. *(–)-Gossypol inhibited cell viability and colony formation in MAT-LyLu and MLL cells. (A) Cancer cells (4,000 cells/well) grew in 96-well plates for 24 hours, then were exposed to the indicated concentrations of (–)-gossypol or 0.1% ethanol (vehicle control, GP-0) for 24 hours. Each bar represents the mean±SD of six wells. The asterisks show significant differences from the control group ($p<0.05$). (B) Comparison of colony formation among different doses of gossypol in MAT-LyLu and MLL cells. Data represent the mean±SD of triplicate determinations from a single experiment, representive of three conducted. Vehicle control was significantly different from (–)-gossypol, *$p<0.05$ by ANOVA. (C) Representative colony photographs of MAT-LyLu and MLL cells in soft agar.*

was firstly performed. Data showed that (–)-gossypol treatment reduced Bcl-2 and Bcl-xL protein expression in a time- and dose-dependent manners in both MAT-LyLu and MLL cells. In contrast, (–)-gossypol treatment elevated nm23-H1 expression in a dose-dependent manner in both MAT-LyLu and MLL cells. Moreover, nm23-H1 proteins were up-regulated by (–)-gossypol when incubation time increased (Figure 5). These current findings were also consistent with previous results (12) in which MLL cells possess lower steady state levels of *nm23* mRNA than those of MAT-LyLu cells and might be correlated with (–)-gossypol-reduced *in vitro* invasive ability.

Discussion

MAT-LyLu was derived from an adult malignant rat prostate tumor obtained from a 22-month-old inbred Copenhagen rat (21). MAT-LyLu cells are fast growing, androgen-independent, highly metastatic and simulate stages II-IV of human prostate cancer. In the current study, the differences between the parental MAT-LyLu cells and the isolated subline MLL cells were initially compared. Results showed that the doubling times of MAT-LyLu and MLL cells were 10.4 and 8.7 hours, respectively, which indicated that MLL cells grew faster than MAT-LyLu cells. The colony forming capacity of MLL cells displayed larger colony size and more colony numbers than those of MAT-LyLu cells. These fast growth rates of MLL cells might be associated with the higher expression of cell cycle regulators such as cyclin-D1, cyclin-D3 and CDK6.

These malignant MAT-LyLu and MLL cells had shorter doubling time than normal cells, indicating that these cells were more active in replication. Cell growth is accompanied by the coordinated expression of multiple genes and pathways, including those required for different phases of cell cycle progression. Cyclin-D1 is induced early in the G_1 phase of the cell cycle and cyclin-D1 with cyclin-dependent kinase (CDK) complexes are important for phosphorylation

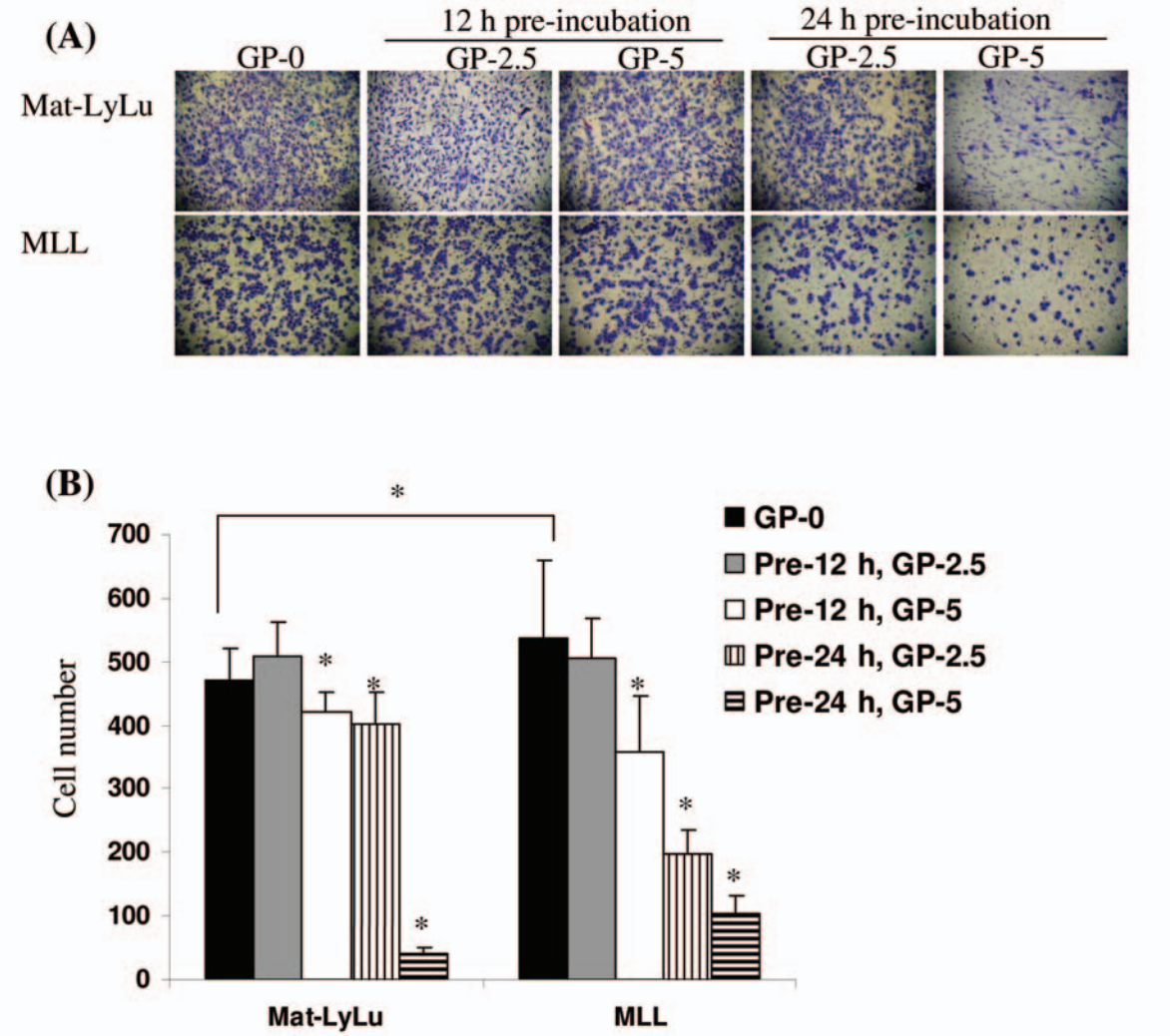

Figure 3. *Effects of gossypol on invasion of MAT-LyLu and MLL cells. (A) Representative photographs of the stained inserts. (B) The number of cells per culture data represent the mean±SD of triplicate determinations from a single experiment, representive of three conducted. Vehicle control was significantly different from gossypol, *p<0.05 by ANOVA.*

of several key substrates involved in cell proliferation including retinoblastoma (Rb) protein and other pocket proteins (22). Amplification or overexpression of cyclin-D1 plays pivotal roles in the development of a subset of human cancer types including parathyroid adenoma, breast, colon, lymphoma, melanoma and prostate (22). This and other studies have found that gossypol arrested the cell cycle at G_0/G_1 phase and modulated cell growth by the inhibition of Rb and cyclin-D1 expression in cancer cells (17, 23, 24). Thus, (–)-gossypol-reduced replication resulted in inhibition of cell viability and colony formation, which might be linked with anti-metastasis activity of (–)-gossypol in MAT-LyLu and MLL cells.

Previous findings indicated that (–)-gossypol could serve as a chemopreventive and chemotherapeutic agent through enhancing apoptosis, down-regulating Bcl-2 and Bcl-xL, and activating caspase activity in cancer cells (16, 17). The Bcl-2 family of proteins constitutes a critical intracellular checkpoint of apoptosis (25) and the Bcl-2 and Bcl-xL proteins possess anti-apoptotic ability. The present data found that MLL cells illustrated higher penetration than MAT-LyLu cells when cells were grown on Matrigel-coated membranes but not on uncoated membranes. One of the reasons might be associated with higher expression of Bcl-xL protein in MLL cells, since overexpression of Bcl-xL in breast cancer cells has been shown to promote metastasis (26, 27). This Bcl-xL expression in breast cancer cells could be linked with inducing resistance to apoptosis against cytokines, increasing cell survival in circulation, and enhancing anchorage-independent growth (26). Although MLL had lower expression of Bcl-2 protein than that of MAT-LyLu cells, Bcl-2 protein also enhances metastasis.

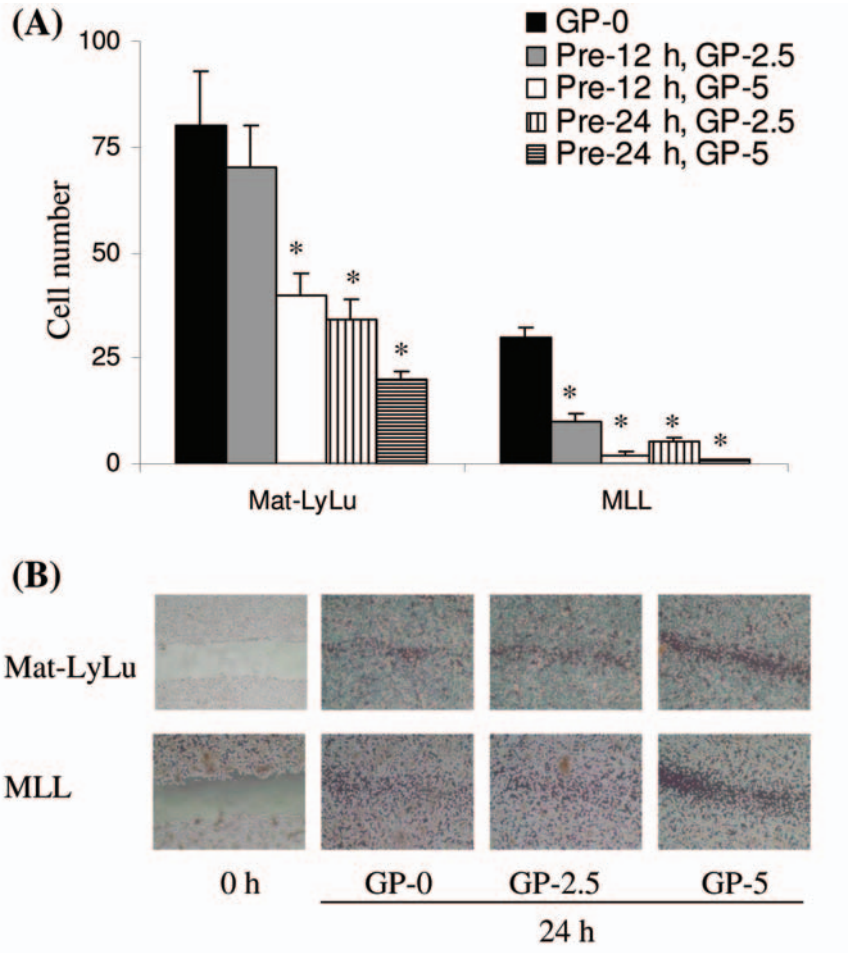

Figure 4. *Effect of (–)-gossypol on cell migration and movement of MAT-LyLu and MLL cells. (A) (–)-Gossypol reduced cell migration ability in MAT-LyLu and MLL cells. Data represent the mean±SD of triplicate determinations from a single experiment, representive of three conducted. Vehicle control was significantly different from gossypol, •p<0.05 by ANOVA. (B) Representative photographs of the cell movement.*

Recently, cells engineered to overexpress Bcl-2 were capable of increasing the *in vitro* invasion capability and the *in vivo* lung metastatic potential in non-small cell lung cancer cells (NSCLC) (28). The promotion of the invasiveness and metastatic potential of NSCLC cells by Bcl-2 showed a strong correlation with the induction and activation of MMP-2: Bcl-2 induced transcription factor activator protein 1 to bind on to the activation site of the MMP-2 promoter (28).

Gossypol has been found to bind to Bcl-2 and Bcl-xL in computer-assisted molecular modeling and fluorescence-polarization assays and is able to bind with the BH3 binding pocket of Bcl-xL in NMR imaging studies (29). Previous results found that (±)-gossypol showed anti-proliferative and anti-metastatic effects on Dunning prostate cell-bearing Copenhagen rats and (±)-gossypol reduced *in vivo* tumor growth and incidence of lung and lymph node metastasis (11). Recent work showed that (±)-gossypol induced dose-dependent inhibition of invasive activity when MAT-LyLu and MLL cells were susceptible to (±)-gossypol (12). More recently, (–)-gossypol was found to inhibit cell growth and to induce apoptosis in overexpressing Bcl-2 or Bcl-xL cancer cells and was able to act directly on the mitochondria to release cytochrome *c* (30). This, and previous (16) studies demonstrated that (–)-gossypol inhibited Bcl-2 and Bcl-xL expression in prostate cancer cells which might be associated with the anti-metastatic capacity of (–)-gossypol in MAT-LyLu and MLL cells. Metastasis enhancement by Bcl-2 and/or Bcl-xL was associated with its survival ability since metastasis is a serial process in which cancer cells survive in the circulation, in a distant organ and then initiate extravasation and colonization (3). (–)-Gossypol inhibited Bcl-2 and Bcl-xL protein expression in cancer cells indicating that (–)-gossypol was linked to a reduction of cell survival and promotion of apoptosis. It was found that anti-sense oligonucleotides down-regulated Bcl-xL protein and markedly increased the sensitivity of prostate cancer cells to

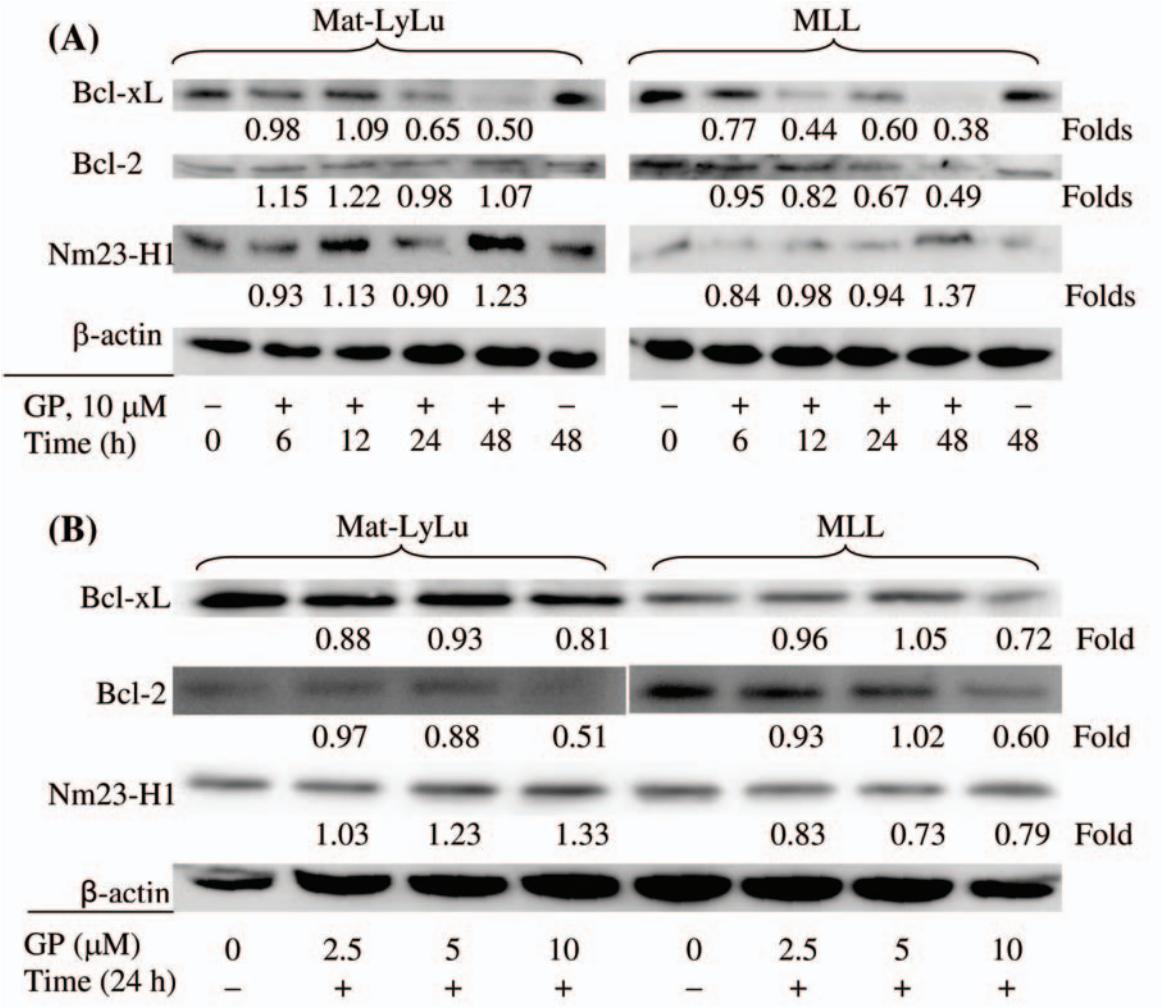

Figure 5. *Effects of (–)-gossypol treatments on nm23-H1, Bcl-2 and Bcl-xL protein expression in MAT-LyLu and MLL cells. (A) Mat-LyLu and MLL cells were grew in RMPI-1640 medium with 5% FBS and then incubated with (–)-gossypol (10 μM) or not for up to 48 hours. (B) MAT-LyLu and MLL cells were treated with (2.5-10 μM) or without (–)-gossypol for 24 hours. The numbers indicate the intensity of protein relative to β-actin (loading control).*

cytotoxic chemotherapeutic agents (31). Another approach using small molecular inhibitors of anti-apoptotic proteins, such as HA14-1, BH3Is and ABT-737, interacted with Bcl-2 and/or Bcl-xL to directly or indirectly induce apoptosis (32, 33, 34). To sum up, (–)-gossypol served as a small molecular inhibitor, suppressed Bcl-2 and Bcl-xL protein expression, decreased cell viability and thus reducing metastasis.

In several clinical trials, gossypol has been demonstrated to meet toxicity criteria and to be well tolerated in patients with metastatic adrenal cancer (35), malignant gliomas (36, 37) and refractory metastatic breast cancer (24), with an antitumor activity at achievable doses (24). It was shown that gossypol inhibited metastatic ability in the prostate metastatic cancer cells *in vitro* (12). The possible anti-metastasis effect of (–)-gossypol might be through up-regulation of metastatic suppressor genes. One of the metastatic suppressors is nm23 which has a nucleoside diphosphate (NDP) kinase. Of the eight human *nm23* genes, the *H1* gene is most closely correlated to the metastatic phenotype in human breast, colorectal and ovarian carcinomas (3). In DU-145 human prostate cancer cells, nm23-H1 expressions were associated with *in vitro* suppression including colonization in soft agar and adhesion to extracellular matrix components, but not growth rates (38). Several reports suggested that the NDP kinase activity of nm23-H1 is independent of its metastasis-suppressive effects in cancer cells and metastasis-suppressing activity of nm23-H1 might depend on its oligomeric structure (39, 40). These results showed that MAT-LyLu expressed higher nm23-H1 protein than MLL cells, which might be associated with the higher penetration of MLL cells than

MAT-LyLu cells in Matrigel-coated membranes. The present findings indicated that (–)-gossypol induced nm23-H1 protein expression in a dose- and time-dependent manner. This induction of nm23-H1 expression is linked with the anti-metastatic ability of (–)-gossypol which inhibited *in vitro* invasion, cell migration and cell movement.

In summary, these studies demonstrate that MAT-LyLu and MLL cells display a similar effect in response to (–)-gossypol treatment. (–)-Gossypol plays a role in metastasis suppression and its mechanism of action in this cell line model may involve down-regulation of Bcl-2 and Bcl-xL protein but up-regulation of nm23-H1 protein. This study highlights the anti-metastatic ability of (–)-gossypol that could have chemotheraputic benefits for prostate cancer patients.

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